AN ABSTRACT OF THE THESIS OF

Kenan G. Heppe for the degree of Honors Baccalaureate of Arts in Chemistry presented on May 26, 2011. Title: Evaluation of Bacterial F_1F_O-ATPase Inhibitors for Synergistic Effects.

Abstract Approved: _____
Dr. Mark Zabriskie

F_1F_O-ATP synthase targeting antimicrobials apoptolidin 1 (A1), (+)-*erythro*-mefloquine, and oligomycin were tested in vitro for synergistic minimum inhibitory concentrations against *Staphylococcus aureus*. Synergy was strictly defined as inhibition by at most 25% the MIC of each participating drug. Ampicillin, with an MIC of 0.8 ug/mL, served as a negative control. (+)-*erythro*-Mefloquine displayed an MIC of 12.5 ug/mL, while oligomycin had an MIC of 1.25 mg/mL. Apoptolidin A1 consistently hindered, yet never completely inhibited, the growth of *S. aureus*. In combination, (+)-*erythro*-mefloquine and apoptolidin A1 yielded an indifferent result. Oligomycin with (+)-*erythro*-mefloquine displayed an antagonistic relationship. One trial of (+)-*erythro*-mefloquine with ampicillin suggested an additive relationship might be present.

Evaluation of Bacterial F_1F_O-ATPase Inhibitors for Synergistic Effects

by

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Head, School of Chemistry

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Dean, University Honors College

I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

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Kenan G. Heppe, author

Acknowledgements

First and foremost I must give thanks to my best childhood friend, Michael Christ, for awakening an understanding that anything can be achieved with enough willpower. I will forever be in debt to the wisdom he displayed at such a young age, and his courage to share it with me.

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To Miracle, my cat.

Evaluation of Bacterial F_1F_O-ATPase Inhibitors for Synergistic Effects

Background

Industrialized antibiotic history commenced in 1929 when Alexander Fleming published his seminal paper in the *British Journal of Experimental Pathology* on the "mold extract" from *Penicillium* as a germ-killing compound.[1] The potential of this unexpected discovery was exploited and received so much acclaim it was called the "miracle drug." The subsequent "Golden Age of Antibiotics" equipped physicians with a large number of "weapons" to combat bacterial diseases.[1]

Doctors were saving people with pneumonia and other dreaded diseases, such as tuberculosis, while also restructuring the foundation on which much of modern medicine rests. Antibiotics made routine surgery feasible. They protected cancer patients that had been rendered temporarily susceptible to infection after chemotherapy. They even cured ulcers, which had been considered chronic conditions. They have also been extended to agriculture, preventing infection and promoting animal growth.[2] However, we have arrived at a largely unanticipated paradox: severe illnesses have re-emerged while pathogens have become resistant to many antibiotics.

Nothing can be done to prevent to emergence of resistance, for it is inherent in natural selection and begins to develop as soon as an antibiotic is used.[3] Therefore, preventing the spread of resistant bacteria is the only way to combat the problem. Before taking preventative measures, we must first understand the three types of antimicrobial resistance: intrinsic, mutational, and acquired.

The Big Three

Some microorganisms are inherently insensitive to certain classes of antimicrobial compounds, which categorize them as intrinsically resistant. The antibacterial agent must be able to penetrate the bacterial surface and reach the target in its active form.[4] Therefore, one may possess an antibiotic that easily destroys a target, but an impermeable cell wall or biofilm could hinder any chance of success.

There are two fundamental structures of the Gram-(-) cell that lead to impermeability: a lipopolysaccharide outer membrane and the expression of outer membrane proteins, porins that restrict inward flow of antibiotics and biocides.[5] Proteins and lipopolysaccharide (LPS) are critical in sustaining the stability of the outer membrane (OM) of Gram-(-) bacteria as a permeability barrier. The core region of the LPS is strongly negatively charged and functions as a selective permeability barrier for negatively charged antibiotics resulting in decreased susceptibility.[5]

Because of the permeability barrier provided by the OM, Gram-(-) bacteria are intrinsically resistant to many hydrophobic antibiotics. By comparison, a similar target in Gram-(+) bacteria is exceedingly attainable because the almost-impermeable membrane doesn't exist.[6]

Biofilms are sessile bacterial communities irreversibly attached to a substrate and enclosed in a matrix of extracellular polymeric substances, the glycocalyx.[7] Resistance is attributable to a growth rate significantly slower than that of planktonic cells, the physiological state of the microorganism associated with expression of genes responding to stress, and delayed penetration and interaction of the antibiotic through the extracellular glycocalyx.[5]

Many organisms are not intrinsically resistant to antimicrobial agents. Howbeit, emergence of mutations in nucleic acids is one of the major factors underlying evolution, providing the working material for natural selection. Due to short generation times, mutations can emerge and accumulate rapidly, effecting significant phenotypic changes perceivably in real-time. Among these changes are those associated with antibiotic resistance.[8]

Mutation rate is not a trivial characteristic reflection of specific bacterial species-antibiotic association. Rather, the probability of the emergence of antimicrobial mutants is a complex phenomenon in which the genetics, physiology, historical behavior of bacterial populations, and the physical structure of the experimental medium play major roles.[9] It is henceforth assumed that the reader understands all circumstances that induce mutation (independent, cooperative, and adaptive) and how pre-existing conditions will favor mutant strains. For the sake of clarity, any further discussion regarding mutation corresponds to selective pressure induced by antibiotics.

Under favorable laboratory conditions, bacterial populations will double in regular intervals: growing as 2^n where "n" = number of generations.[10] This represents exponential growth, which is the fastest of four phases observed in nature: lag, exponential, stationary, death. Some species' generation (doubling) time is a mere 17 minutes, such as *Escherichia coli*. This project was centered around *Staphylococcus aureus*, which has a generation time of about half an hour.[10] In general, the mutation rate in bacteria is rougly 10^{-9} per base pair per generation.[11] *S. aureus* has about 3×10^6 base pairs.[12] Therefore, *S. aureus* will evince about

$$\frac{(1\ x\ 10^{-9}\ mutations)}{(base\ pair)\ (generation)} \bullet \frac{(3\ x\ 10^{6} base\ pairs)}{(S.aureus\ cell)} = 3 \text{ x } 10^{-3} \text{ mutations per } S.\ aureus \text{ cell}$$

generation. Thus, roughly three mutants appear for every thousand cells.

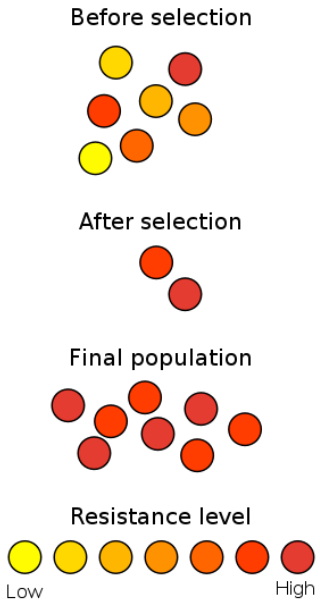

Figure 1: Propagation of antibiotic resistant bacteria.

Due to rapid growth and high numbers (10^{8}-10^{10} organisms per infection), mutations are common. Most sensitive first generation cells will die during antibiotic treatment, but eventually a resistant mutant strain will monopolize the population. Thus, successive generations will exhibit resistance.

Furthermore, genes for resistance can be exchanged between strains and species of bacteria. This third type, acquired resistance, can occur by the processes of lateral horizontal gene transmission (HGT). This is the process whereby genetic material contained in small packets of DNA can be transferred between individuals of alike or different bacterial species. Mechanisms of HGT can be sorted into three categories: transduction, transformation, and conjugation.[10]

Transduction is the process of bacteriophages transferring DNA between two similar bacteria. Transformation occurs when the bacteria take up parts of the DNA from an external environment. The DNA is normally present in the external environment as a result of death and lysis of other bacteria. Finally, conjugation occurs when there is direct contact between two bacteria (which don't have to be closely related) and the transfer of plasmids takes place.[18]

The Approach

With so many mechanisms of resistance available to the bacterial kingdom, it is difficult to predict the most efficient way of inhibiting growth. The novel approach attempted in this study revolves around adenosine triphosphate (ATP) synthesis. ATP is considered by biologists to be the energy currency of life, for essentially all the physiological mechanisms that require energy for operation obtain it directly from stored ATP.[13] Therefore, if ATP production were prevented in a bacterial cell then it would perish.

Only minor differences exist between the ATP production complexes of bacteria and mitochondria. In bacteria, the ATPase and electron transport chain are located inside the cytoplasmic membrane; between the hydrophobic tails of the phospholipid membrane's inner and outer wall.[14] Sugars such as glucose are metabolized, leading to the transport of protons from the matrix across the inner membrane, storing energy in the form of a transmembrane electrochemical gradient. Protons then travel back across the gradient through the ATP synthase enzyme, providing enough energy for inorganic phosphate and adenosine diphosphate to form ATP.[15]

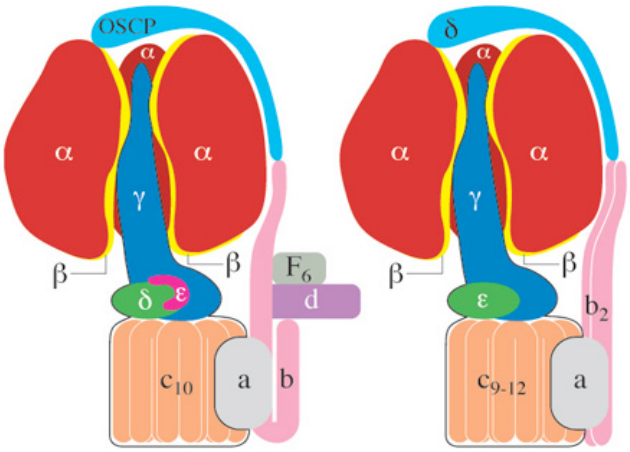

Figure 2: Structural comparison of mitochondrial (left) and bacterial (right) F_1F_O-ATPase.

Although strikingly similar, the slight differences in amino acid composition and structure of each enzyme are what permit drug selectivity.

Mammalian mitochondrial F_1F_O-ATPase is slightly more complicated than the bacterial enzyme, with a few additional subunits.[16]

Mitochondrial ATP synthase is a huge molecular complex (>500,000 daltons) embedded in the inner membrane. Its role is to synthesize ATP from adenosine diphosphate (ADP) and inorganic phosphate. It is fueled by the energy of protons moving down an electrochemical gradient. The overall reaction is as follows:

$$ADP + P_i \rightarrow ATP$$

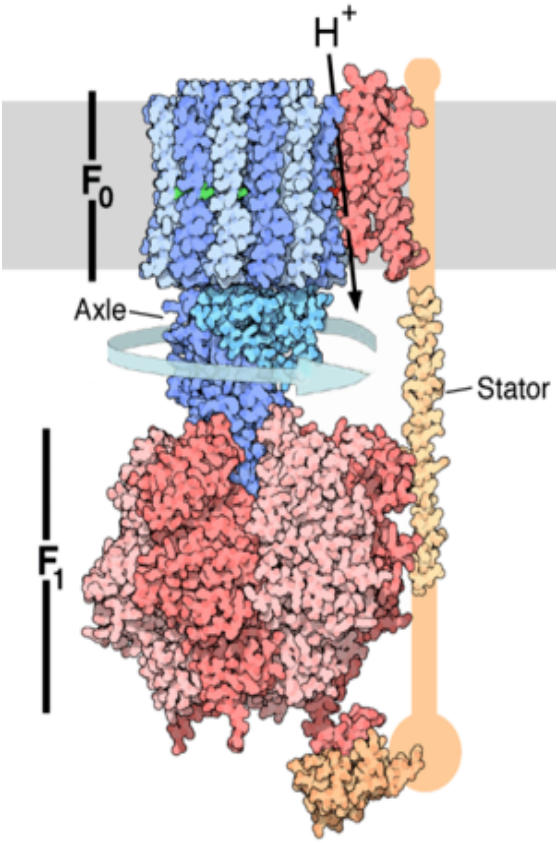

Figure 3: Functional overview of mammalian mitochondrial F_1F_O-ATPase.

In Fig. 3, the F_O portion is shown within the grey membrane, whereas the F_1 portion is below the membrane, inside the matrix of the mitochondria. In bacteria, F_O is outside the cell while the F_1 portion lies in the cytoplasm. The intact enzyme is commonly called F_1F_O-ATPase. The protons that have accumulated in the membrane space enter the F_O complex and exit into the matrix. The energy released as they travel rotates F_O and the stalk at about 6000 rpm in a clockwise direction. The rotation induces repeating conformational change in the head proteins that enable the conversion of ADP and P_i into ATP.[17]

The anti-fungal activities of macrolide natural products, such as oligomycin, that target the mitochondrial F_1F_O-ATPase are well documented.[18] In fact, the F_O subunit is commonly labeled with a misnomer by using a zero instead of an "O," for the "O" represents the oligomycin-binding fraction.[19] This proton channel is necessary for the oxidative phosphorylation that ATPase requires. A mixture of oligomycin analogues A, B, and C were used in this experiment.

Oligomycin	R^1	R^2	R^3	R^4	R^5
A	CH_3	H	OH	H,H	CH_3
B	CH_3	H	OH	O	CH_3
C	CH_3	H	H	H,H	CH_3

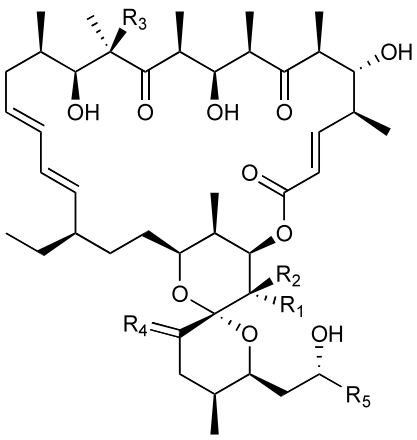

Figure 4: Structures of the oligomycins.

Mefloquine is a synthetic agent used to prevent and treat malaria. It was found to be 10-fold more active than optochin and about 200-fold more active than quinine in inhibiting both the growth and the ATPase activities of laboratory pneumococcal strain R6.[20] Mefloquine has also been found to be bactericidal against Gram-(+) bacteria including staphylococci and enterococci.[21] Therefore, it may have the same effect on ATPase activities in staphylococci.

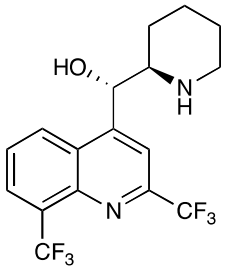

Figure 5: Structure o
(+)-erythro-mefloquin

The apoptolidins are macrolide natural product antibiotics originally identified on the basis of their ability to selectively kill E1A and E1A/E1B19K transformed rat glial cells while not killing untransformed glial cells. It has been demonstrated that apoptolidin

is a potent inhibitor of F_1F_O-ATPase activity in intact yeast mitochondria.[18] It resides within a small family of polyketide inhibitors of F_1F_O-ATPase, and is among the top 0.1% most cell line selective cytotoxic agents of 37,000 molecules tested against the 60 human cancer cell lines of the National Cancer Institute.[22]

Under slightly acidic conditions, apoptolidin A can rearrange into the 21-membered macrolactone isoapoptolidin by a O19-O20 acyl shift.[23] This isomerization renders the molecule inactive. Therefore we sought to pursue a different analogue.

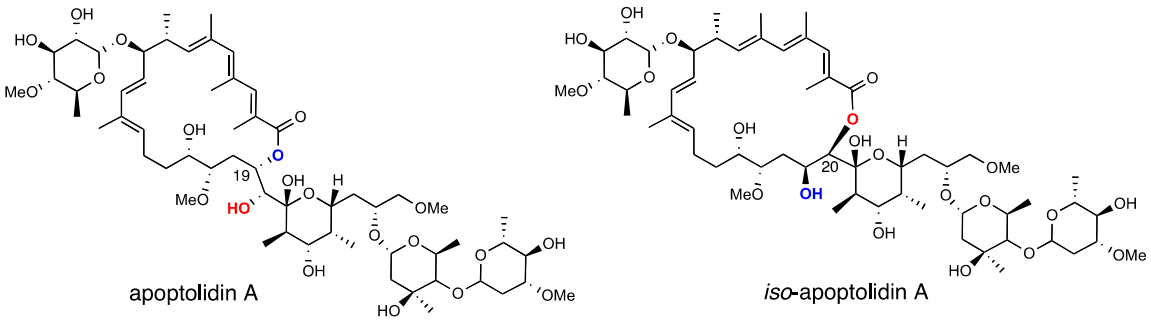

Figure 6: Structure of apoptolidin A and iso-apoptolidin A.

Recently, another analogue of apoptolidin was isolated and structurally characterized. Apoptolidin C differs from the A analogue by the absence of the OH groups at C16 and C20.[23] There is great interest in this analogue because it is stable with respect to isomerization.

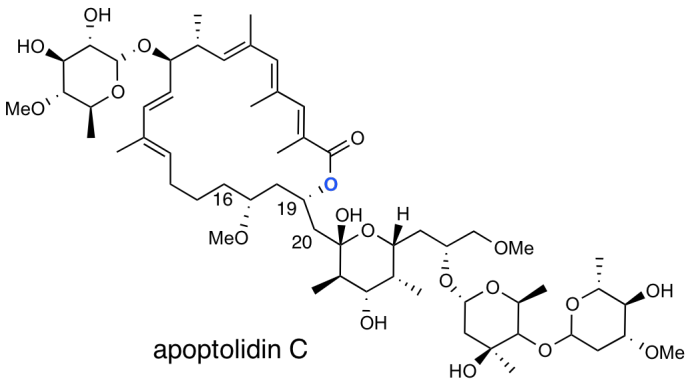

Figure 7: Structure of apoptolidin C.

Staphylococcus aureus was the focus of the study, while ATP synthase was the target. With three known inhibitors of F_1F_O-ATPase available, our goal was to see how combinations of compounds in varying concentrations would compare to monotherapy minimum inhibitory concentrations (MICs).

Synergy

Combining two or more antimicrobial agents in the treatment of bacterial infections has become ordinary practice, particularly in immunosuppressed hosts. The premise of this approach is that the combination will induce an effect greater than what would be predicted through monotherapy.[24]

Such a thing is quite plausible if one considers the targets and mechanisms of the agents. If two antimicrobials target F_1F_O-ATPase yet bind to different subunits, one may make the other's job extremely easy. But if two agents bind to the same target, they will compete for the same goal and the result may possibly be worse (antagonism).

Most methods of testing for synergy have compared MICs of two agents alone and in combination, as in the checkerboard technique. Another method assesses synergy by comparing rates of killing with a given bacterial inoculum for antibiotics individually and in combination (in vitro killing curves).[25]

Although multiple methods for detecting synergy exist, the killing curve method would have taken more training time. Thus, on the simple premise of convenience we were led to the checkerboard technique: within a 96-well plate we would hold the concentration of drug A constant while varying B, and in a separate row vice versa. Additionally, some rows would serve as controls and blanks.

How much reduction in drug concentration is necessary to declare synergy? Without standardization, subjective interpretation may ensue. Thus, criterion for synergy evaluation must be strictly defined. For this project, combinations were considered to be synergistic when the concentration of each agent in combination was reduced to <25% of the amount required for each agent alone to inhibit growth.[25] On the other hand, the combination was considered to be antagonistic if growth occurred above the MIC of either drug in question. If inhibition occurred at the MIC of either drug, the combination was considered indifferent. An additive effect would mean that an MIC was reduced, but not so much as to be synergistic.

Method

Materials

Table 1: Experimental products and respective sources.

Product	Source
(+)-*erythro*-mefloquine	Gift from Dr. Takashi Suyama
96-well Polysyrene Plate	Cellstar Cat. No. 655 185
Ampicillin	Sigma-Aldrich
Apoptolidin	Gift from Dr. Noer Kasanah
Centrifuge Tubes (15 mL)	VWR Cat. No. 89004-368
HPLC Programmable Detector	Beckman System Gold Detector #166
HPLC Programmable Solvent Module	Beckman System Gold Pump #125
Incubator Shaker	Barnstead Labline MaxQ 4000 Lot: 61412003
Laminar Flow Hood	Labconco Class II Type A2 Purifier
Microcentrifuge Tubes	VWR Cat. No. 87003-290
Microplate Reader	SpectraMax 190 (MTX Lab Systems, Inc.)
Oligomycin	Sigma-Aldrich #04876 (25 mg)
pH Meter	Mettler Toledo MP220 pH Meter
Pipet Tips	Bio Plas, Inc. Cat. No. 3700GL
Sonicator	Branson UltraSonic Cleaner #26373
Speed Vacuum (speedvac)	Thermo Scientific – Savant Speed #131dda
Staphylococcus Aureus	American Type Culture Collection
Tryptic Soy Broth	Cellgro (Media Tech, Inc.)

Product Purification

Dr. Noer Kasanah provided our team with a sample of crude apoptolidin. To isolate and purify the analogs A and C we used a Beckman System Gold HPLC. The flow rate was set to 5 μL/min and wavelength λ to 254 nm. The samples with retention time of 15 & 18 minutes were apoptolidin A. The 23-minute peak corresponded to apoptolidin C. After every five runs the similar compounds were consolidated and dried in the centrifugal concentrator, while finally being placed into vials for later use. The solvent used for apoptolidin was 50% aq. MeOH.

Minimum Inhibitory Concentration (MIC)

Before using the apoptolidin samples or testing for synergy, it was necessary to learn the following techniques with less expensive products. First, we would make sure our MICs for known antibiotics would match literature, and then we would proceed to the unknown work.

Many boxes were filled with 200 μL pipet tips. Tryptic Soy Broth (TSB) was made according to the manufacturer's directions and brought to pH 7.3 ± .2. The tips and broth were autoclaved separately for thirty minutes. Samples of *S. aureus* were used to make a streak plate on TSB agar. The plate was parafilmed and placed in a 37 °C oven for sixteen hours. The sterile tips and broth rested in a laminar flow hood.

After growth of *S. aureus* on the streak plate was confirmed, three 15 mL centrifuge tubes were obtained. Five mL of TSB broth was transferred to each tube. Using sterile loops, one pure *S. aureus* colony was placed in one of the tubes, two into another, and three into the final. The inoculated centrifuge tubes were taken to the incubator shaker set to 37 °C and left for 2-4 hours.

While the bacteria incubated, 1 mg of ampicillin or oligomycin was dissolved into an appropriate solvent to make a 1 mg/mL stock solution. A 2:1 dilution series was made in microcentrifuge tubes with the first tube's drug concentration being 1 mg/mL. This is only because we desired our first well to have a final drug concentration of 100 μg/mL. If other concentrations were desired, appropriate adjustments were performed. A serial dilution was made for each antibiotic of interest in the trial. However, for combination wells one drug is held constant, therefore a stock solution will suffice. TSB broth, antibiotics and their respective solvents were placed in a 96-well polystyrene plate according to a map of the desired layout (See Table 2). This plate was set-aside for the time being (if light sensitive antibiotics were being used, it was covered with aluminum foil).

Following the 2-4 hour incubation period, a separate 96-well plate was retrieved. Inside the hood, 200 μL of the fresh *S. aureus* was placed into a well using a pipet. For each centrifuge tube, two or three wells were inoculated. Using the SpectraMax microplate reader, the turbidity was measured to give an approximation of bacterial cell density. The wavelength λ was set to 600 nm, and using the program's template the corresponding wells were labeled as unknowns. A sample was chosen if its absorbance was between 0.08 and 0.13. According to the Clinical and Laboratory Standards Institute, an absorbance between 0.08 and 0.13 contains approximately 1 to 2 x 10^8 CFU/mL. If outside this range, a dilution in respective sterile medium was performed to obtain an absorbance within this range.[26]

A 25 mL tub was filled with 20 mL of TSB broth. Then, 50 μL of the successful absorbance colonies were placed in the tub. The bath was shaken very gently and the

remaining bacteria were discarded accordingly. Finally, 100 μL of the cultured bacteria were placed into the appropriate wells of the original plate. The plate was taken to the microplate reader for a "Time 0" reading, parafilmed, and placed in a 37 °C oven for sixteen hours. All micropate reader files were saved on an external drive.

The plate was recovered and taken to the microplate reader for another reading. Using the "16-hour" and "Time 0" data, Microsoft Excel was used to process the results. Optical Density (OD) Change was calculated, followed by Average OD change for duplicate negative control wells. Then, a "% Growth" section was made to normalize the data around the negative control, and graphs were made to show the MICs visually.

Table 2: Example checkerboard plate layout.

	1	2	3	4	5	6	7	8	9	10	11	12
A	-	-	-	-	M	M	M	M	M	M		
B	Olg. +M1	Olg. +M2	Olg. +M3	Olg. +M4	Olg. +M5	Olg. +M6	Olg. +M7	Olg. +M8	Olg. +M9	Olg+ M10	B	B
C	Olg. +M1	Olg. +M2	Olg. +M3	Olg. +M4	Olg. +M5	Olg. +M6	Olg. +M7	Olg. +M8	Olg. +M9	Olg+ M10	B	B
D	Amp.	Amp.	Amp.	Amp.	Amp.	Amp.	Amp.	Amp.	Amp.	Amp.	B	B
E	Mfq.	Mfq.	Mfq.	Mfq.	Mfq.	Mfq.	Mfq.	Mfq.	Mfq.	Mfq.	B	B
F	Amp + M1	Amp + M2	Amp + M3	Amp + M4	Amp + M5	Amp + M6	Amp + M7	Amp + M8	Amp + M9	Amp + M10	B	B
G	Amp + M1	Amp + M2	Amp + M3	Amp + M4	Amp + M5	Amp + M6	Amp + M7	Amp + M8	Amp + M9	Amp + M10	B	B
H	Amp + M1	Amp + M2	Amp + M3	Amp + M4	Amp + M5	Amp + M6	Amp + M7	Amp + M8	Amp + M9	Amp + M10	B	B

The negative controls (-) were made up of 20 μL solvent, 80 μL media, and 100 μL cells. The media blanks (M) were 200 μL media. Blanks (B) were 20 μL solvent, and 180 μL media. Oligomycin (Olg.), mefloquine (Mfq.), ampicillin (Amp.), or apoptolidin 1 (A1) were added to cells exclusively (20 μL) or in combination (10 μL each drug).

Results

Apoptolidin A with retention time of 15 minutes was purified, and 6.1 mg was collected and stored in a small vial. Similarly, 2.8 mg of retention time 18 minutes was also stored. The amount of pure apoptolidin C collected was so small that the scale detected no difference between the empty and full vial.

The MIC for various antibiotics was determined in vitro using the microplate method described earlier. Our control antibiotic, ampicillin, displayed an MIC of 0.8 μg/mL.

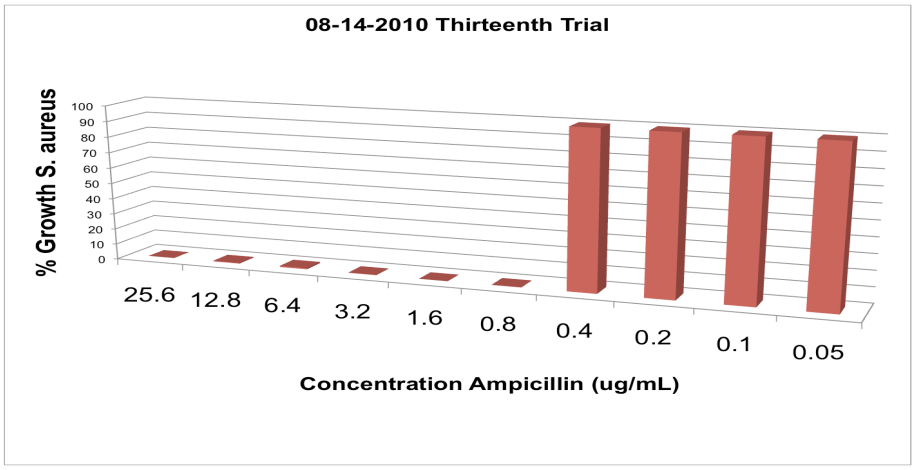

Figure 8: MIC of Ampicillin against *S. aureus*.

The MIC of (+)-*erythro*-mefloquine was determined to be 12.5 ug/mL

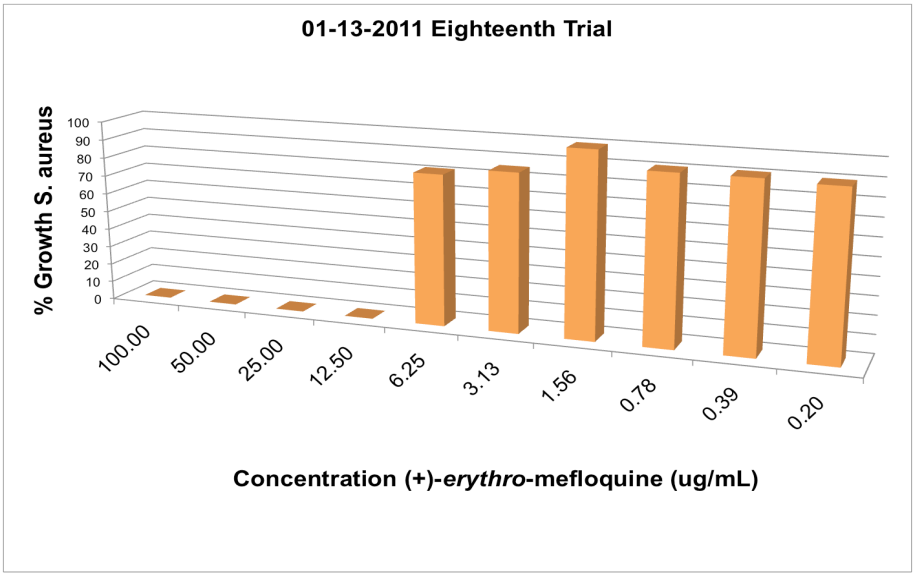

Figure 9: MIC of (+)-*erythro*-mefloquine against *S. aureus*.

Apoptolidin never showed an MIC, but its presence appeared to decrease the amount of *S. aureus* growth.

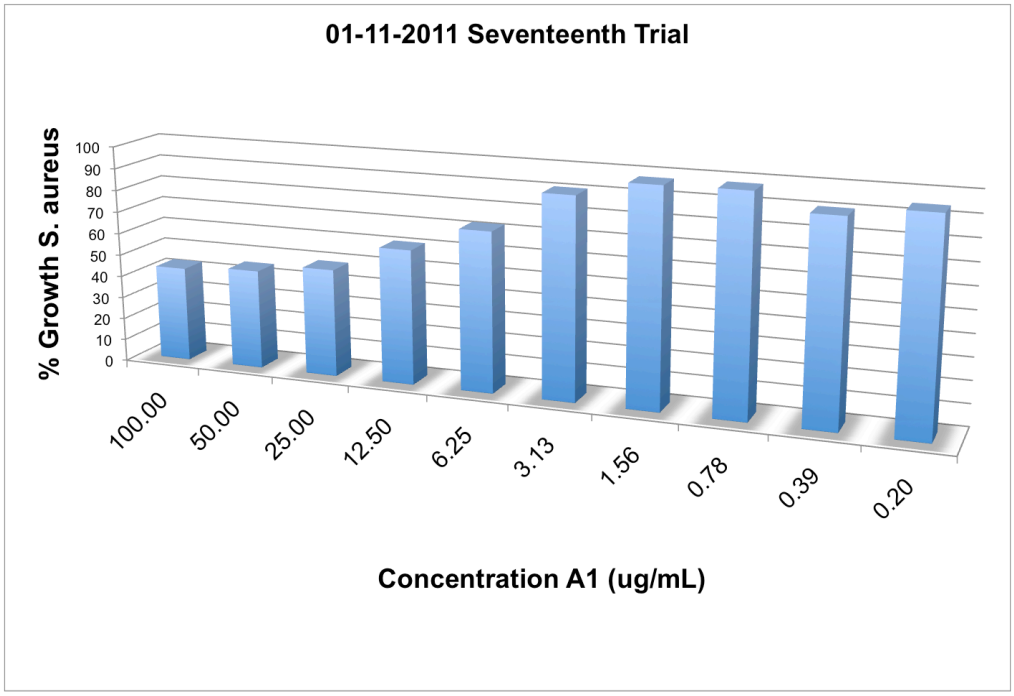

Figure 10: MIC of apoptolidin 1 against *S. aureus*.

Finally, oligomycin had quite a high MIC of 1,024 µg/mL.

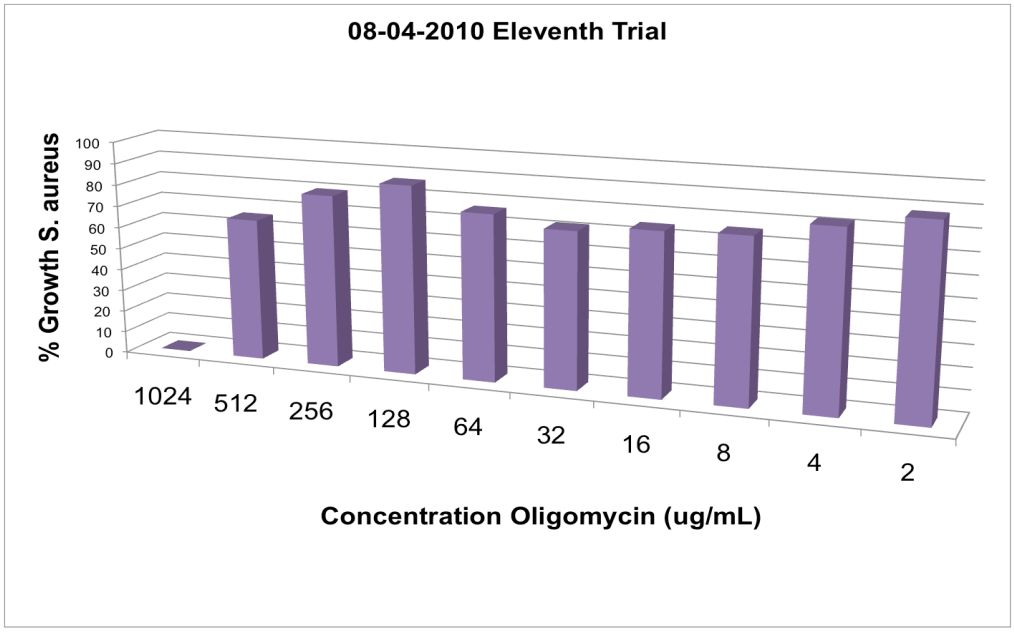

Figure 11: MIC of oligomycin against *S. aureus*.

Apoptolidin and (+)-*erythro*-mefloquine were combined, holding the concentration of A1 constant and varying the concentration of Mfq. No effect was observed with this combination. Inhibition would occur at the regular (+)-*erythro*-mefloquine MIC of 12.5 μg/mL.

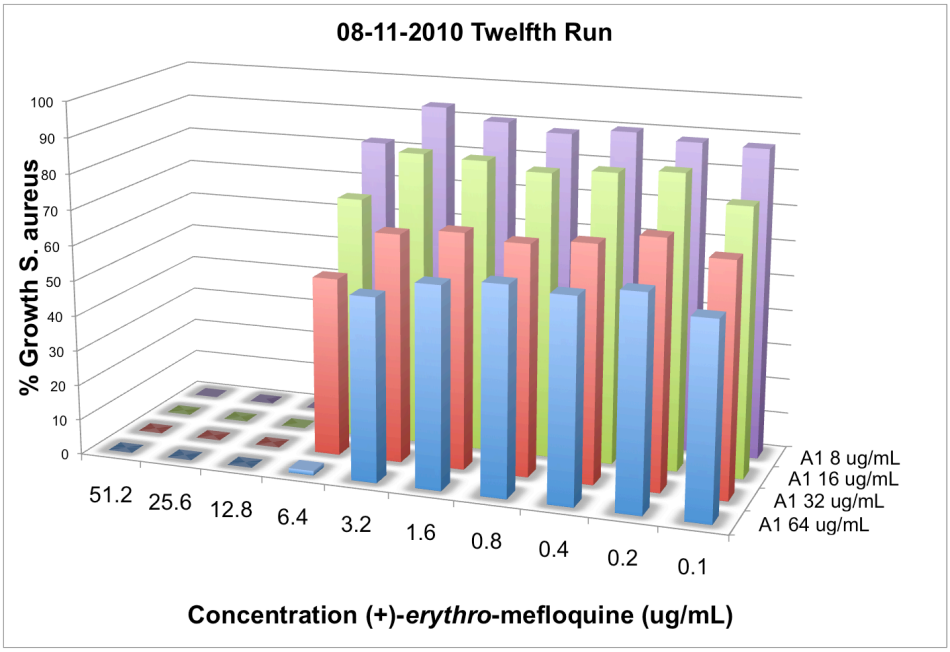

Figure 12: Combination MIC of (+)-*erytho*-mefloquine and apoptolidin 1 against *S. aureus*.

The combination of oligomycin and (+)-*erythro*-mefloquine was tested, and an antagonistic relationship was discovered. While low concentrations of oligomycin were held constant, the MIC of the combination was the normal MIC of (+)-*erythro*-mefloquine. However, when the concentration of oligomycin was increased to 50 μg/mL or 200 μg/mL, inhibition required 50 μg/mL of mefloquine (see Fig. 12 & 13 on following page).

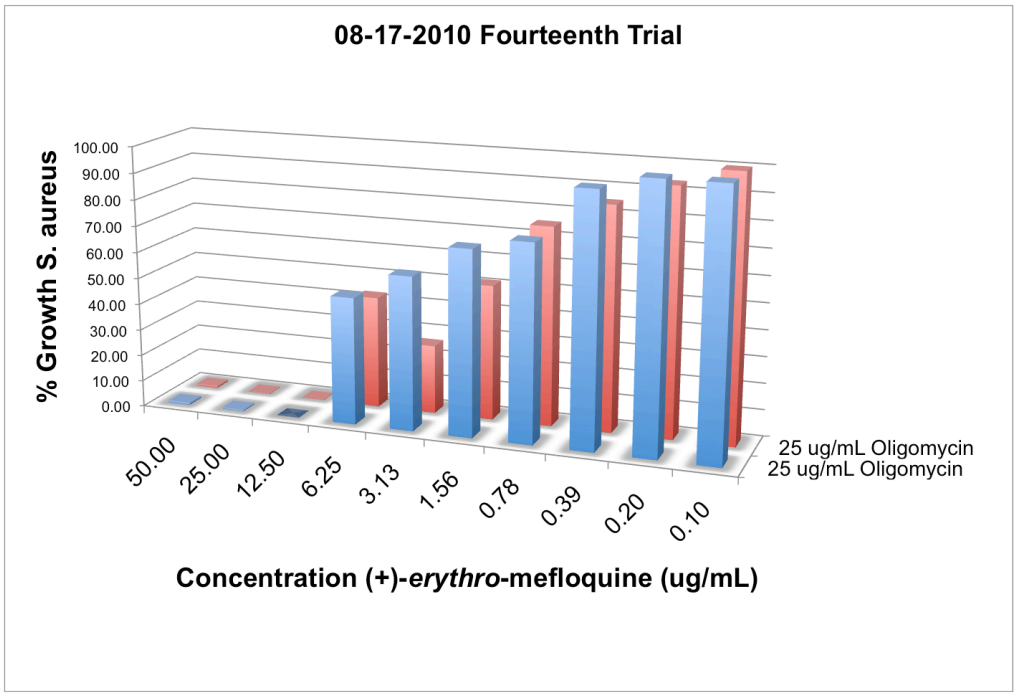

Figure 13: Combination MIC of (+)-*erythro*-mefloquine and 25 ug/mL oligomycin against *S. aureus*.

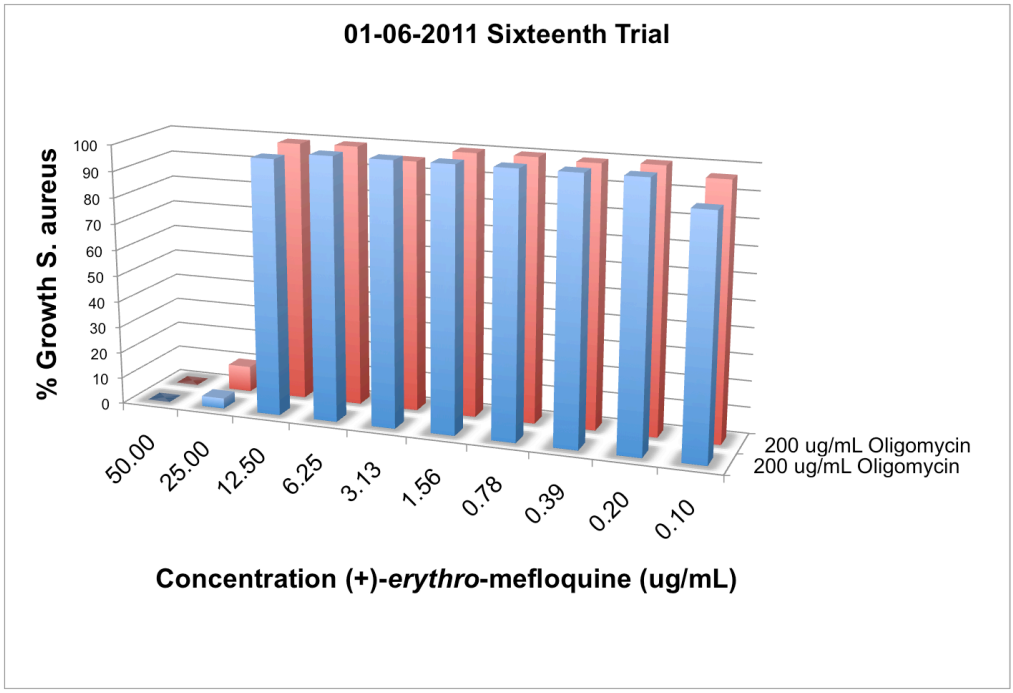

Figure 14: Combination MIC of (+)-*erythro*-mefloquine and 200 ug/mL oligomycin against *S. aureus*.

Ampicillin was also tested in combination with (+)-*erythro*-mefloquine, but again no synergy was found. The data suggest that an antagonistic relationship may exist, but further testing was not conducted.

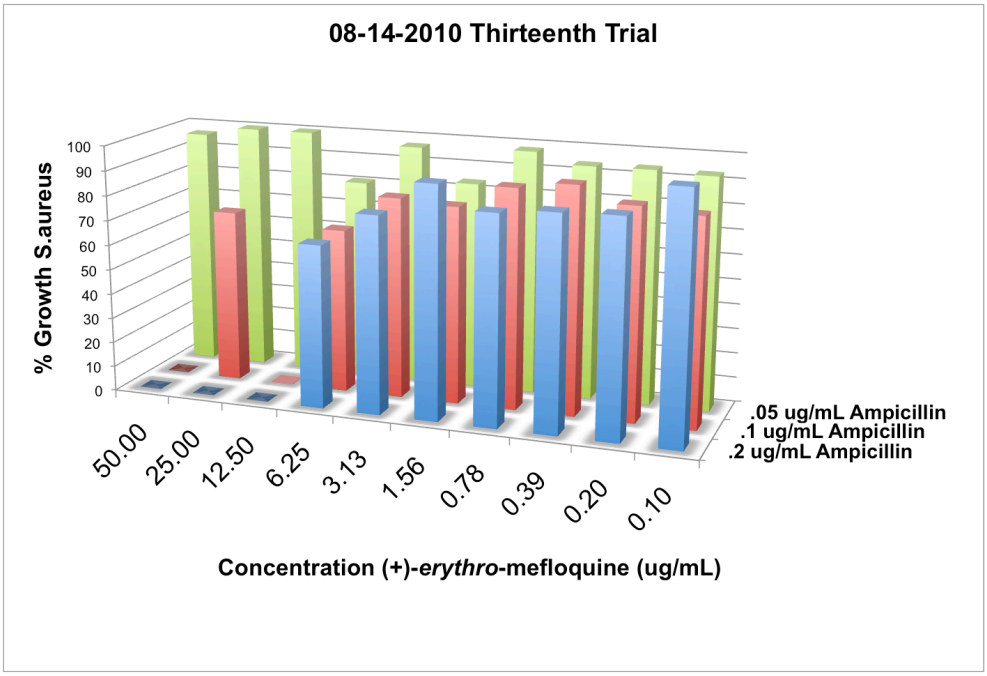

Figure 15: Combination MIC of (+)-*erythro*-mefloquine and ampicillin against *S. aureus*.

Discussion

We hoped that the purification stage would lead to a useful amount of apoptolidin C. However, such a small amount was isolated that the scale couldn't even discern how much was obtained. If the ratio of the size of the container with respect to the sample is too high, the reported mass can be skewed. Because the amount was unknown, we were required to use apoptolidin A1 instead (the mixture of retention times 15 & 18 minutes).

The experimental technique was verified because the MIC of ampicillin agreed consistently with literature. Results for (+)-*erythro*-mefloquine and oligomycin were also constant throughout trials. However, apoptolidin differed from the rest of the drugs

because it never displayed an MIC. Because we were unable to use the stable "C" form of apoptolidin, we were forced to use the analogue that becomes inactive by isomerization. Therefore, it is possible that little drug activity was occurring and growth may have been inhibited simply by the presence of solvent.

Similar observations were made after the first checkerboard experiment between apoptolidin and (+)-*erythro*-mefloquine. The combination MIC while varying the concentration of apoptolidin A1 was basically equal to that of mefloquine alone. However, one visible trend exists: as the concentration of apoptolidin A1 is increased we see a decrease in bacterial growth up until the concentration is 64 ug/mL, and the MIC visibly changes to 6.4 ug/mL (+)-*erythro*-mefloquine. According to our definition of synergy this is an additive effect. Indeed, the MIC of mefloquine was reduced, but the reduction required a significant increase in the concentration of A1. The shortcoming here is that we are unsure whether or not pure apoptolidin C would have made a positive difference.

The combination of oligomycin with mefloquine was even more disappointing. As the concentration of oligomycin was increased, the MIC of (+)-*erythro*-mefloquine also increased. Therefore, according to our definitions, this combination is antagonistic with respect to synergy. It is possible that the least potent drug bound to the enzyme and formed a stable product that decreased the binding affinity of the more potent antimicrobial, resulting in less inhibition. It is also possible that oligomycin and (+)-*erythro*-mefloquine interacted with one another, for example, allowing oligomycin to take mefloquine out of solution.

Up until this point, neither drug was examined at a quarter of its respective MIC. This means that no combination was even close to being synergistic. Out of curiosity, we combined ampicillin with (+)-*erythro*-mefloquine and saw something somewhat interesting. Fig. 16 reveals that low concentrations of ampicillin cause the MIC of (+)-*erythro*-mefloquine to rise, but at 0.2 ug/mL (25% of ampicillin's MIC) it is clear that the combination is equal to (+)-*erythro*-mefloquine strength alone. This implies an additive effect, making it the most successful combination in the study. Because addition was not the focus of this study, the combination of ampicillin and mefloquine was not examined any further.

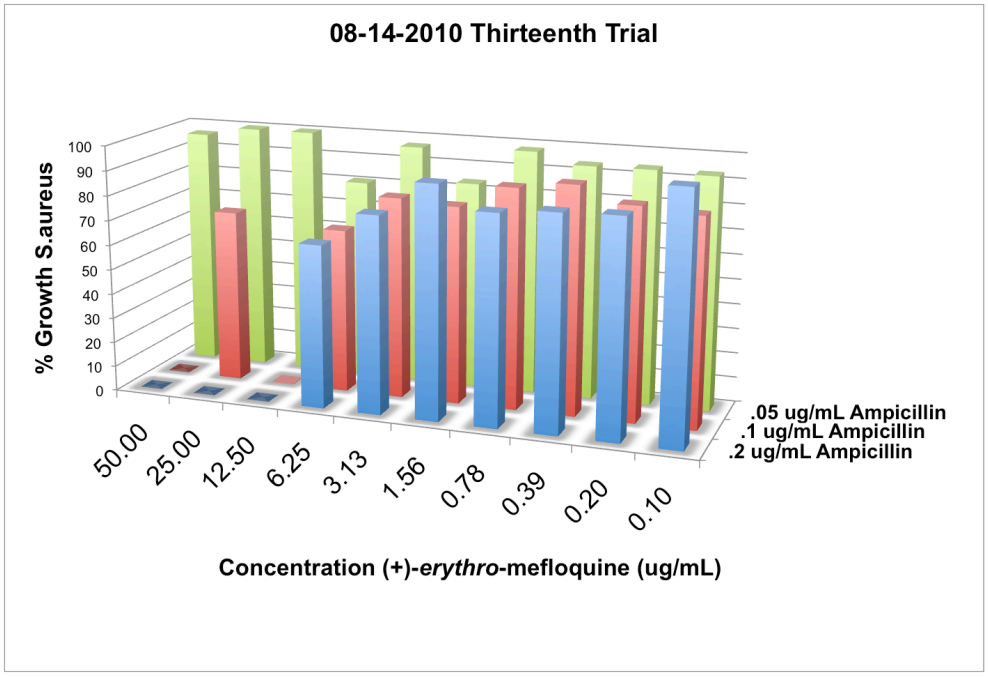

Figure 16: Combination MIC of (+)-*erythro*-mefloquine and ampicillin against *S. aureus*.

Ampicillin prevents the formation of peptidoglycan, an essential building block of the bacterial cell wall.[27] So, it may be very possible that this combination yielded the most positive results because ampicillin made the ATP enzyme more accessible for (+)-

erythro-mefloquine. The other combinations may have been competing with one another for work, causing a negative result.

One large shortcoming may lie in the simplicity of the experiment. Only one criterion for synergy was enforced, and it has been previously shown that when checkerboard and killing-curve techniques are compared, there can be poor correlation in terms of the frequency of strains showing synergy.[25] Thus, stronger arguments for or against synergy can be made only by employing both techniques and producing similar results.

This experiment, although no synergistic combination was found, is very significant. Antibiotic resistance is absolutely one of the biggest issues facing the human race today, and therefore requires enormous attention. The combinations of antimicrobials in this test did not produce favorable results, so without wasting any more time on these couples we can move on to new pairs and ideas. This minor failure should serve as extreme impetus to fund and search for new antibiotics. These pathogenic life forms are ruthless, fast, and tricky. We need to work now, and fast.

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